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INTRODUCTION

Anti-estrogens such as tamoxifen are important therapeutic agents in the treatment and chemoprevention of breast cancers. Other compounds such as phytoestrogens, fatty acid amides such as anandamide and retinoid X receptor (RXR) agonists are also effective against breast cancer in cell lines and in animal models. Because these compounds are unrelated, it has not been appreciated that they might act through a common mechanism. These compounds all share the ability to activate a heterodimer of the steroid and xenobiotic receptor (SXR) and RXR. Our hypothesis is that SXR serves as a common molecular target for some of the anti-proliferative effects of these compounds and that activation of SXR is itself anti-proliferative. To this end, we have found that a constitutively active form of SXR, VP16-SXR is able to slow the growth of transiently transfected breast cancer cells similar to treatment with SXR activators, and we are in the process of constructing stable cell lines with controlled expression of VP16-SXR to confirm this result. We have detected the expression of SXR in ductal carcinomas, but not in normal breast tissue raising the possibility that the presence or absence of SXR may be related to breast cancer treatment outcome.

BODY

Task 1. To determine whether the anti-proliferative effects of phytoestrogens, fatty amides, anti-estrogens and RXR agonists are due to activation of SXR/RXR in breast cancer cells by these compounds. (months 1-12)

Survey breast cancer cell lines for SXR expression.

Our preliminary results showed that SXR.2 is expressed in four breast cancer cell lines out of four tested. These cells include two estrogen receptor (ER) positive lines, MCF-7 and T47D and two ER negative lines MDA-MB231 and MDA-MB437. These four cell lines are adequate for our in vitro analyses and the identification of target genes that will be conducted during years 2 and 3. Therefore, rather than conduct an exhaustive survey of other breast cancer cell lines, which may not themselves be representative of normal breast tissues, we have focused our efforts on localizing SXR mRNA and protein in tissues as described below.

Localization of SXR mRNA and protein in breast biopsies.

SXR protein:

We extensively characterized our anti-SXR antisera for their ability to recognize SXR in cultured cells and in tissue sections. We found that of the three antisera which were raised against peptides derived from SXR, two were able to detect SXR proteins in western blots of either in vitro transcribed/translated SXR or extracts from cells transfected with SXR expression plasmids (anti-124/139 peptide lot #6506 and anti-412/427 peptide). The numbers refer to the starting and ending amino acids of the peptide with respect to full length SXR protein). We tested these two affinity-purified antibodies along with a commercial anti-SXR antibody (PXR N-16, Santa Cruz Biotechnology) for their ability to detect SXR protein in either cultured HepG2 (liver) cells or in sections of human liver, intestine or breast. None of the antisera or purified antibodies was able to localize SXR protein in these tissues despite extensive optimization and testing. SXR mRNA is known to be expressed at very high levels in liver and at high levels in intestine. Therefore, the inability to detect SXR protein in breast tissue suggests that no conclusions can be drawn about the presence of SXR in breast at this time. We infer that these antisera are inadequate for the detection of SXR protein and that a new antiserum must be generated in order to detect SXR protein.

SXR mRNA:

We previously showed that SXR mRNA can be detected in breast cancer cell lines and this is in agreement with a published report (1). Intriguingly, Dotzlaw and colleagues detected SXR mRNA in both tumor tissue and in normal breast (1). We employed quantitative real time RT-PCR analysis (RTQ-PCR) to evaluate whether SXR was expressed in invasive breast carcinomas compared with the corresponding normal tissue (2). We found that SXR mRNA could only be detected in breast carcinomas (20 of 48 samples) but not in the normal breast tissues (2). We next used laser capture microdissection to reliably separate normal breast tissue from breast carcinoma and purified RNA from the resulting tissue fragments. RTQ-PCR analysis showed that SXR mRNA could only be detected in the carcinoma cells but not in surrounding stromal cells (2). We are currently investigating whether the differences between our results and the results of Dotzlaw et al., result from the different populations tested (Western vs. Japanese women) or different methods employed.

Transiently transfect cells with VP16-SXR and DsRed and analyze effects on proliferation after FACS separation of transfected cells.

MCF7 breast cancer cells were transiently transfected with VP16-SXR, VP16 or control vector together with pDsRed. The pDsRed plasmid enables one to separate transfected from untransfected cells using fluorescence activated cell sorting. The transfected cells were then seeded into 96-well plates and grown for 5 days after which CyQuant cell proliferation assays

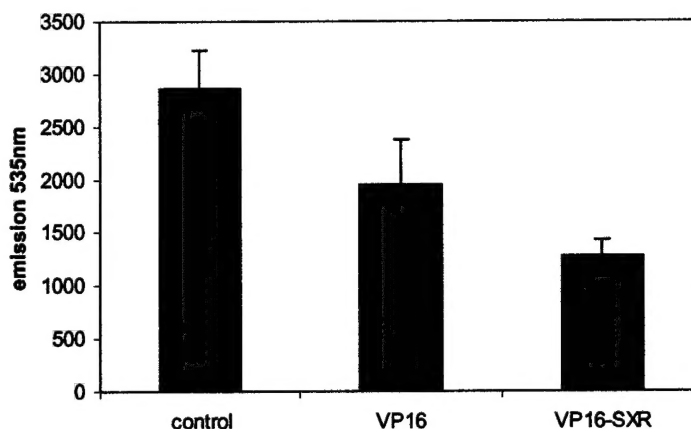


Figure 1 Inhibition of MCF7 cell proliferation by transfected VP16SXR or control plasmids.

were performed to determine the effects of VP16-SXR on proliferation. As shown in Figure 1, VP16-SXR can lead to substantial inhibition of MCF7 cell proliferation compared with control vector or VP16 expression vector alone. VP16 itself appears to have an inhibitory effect on proliferation of cells; however, VP16-SXR clearly has a further effect on inhibiting proliferation. These positive results suggest that it will be worthwhile to construct cell lines stably expressing VP16-SXR under the control of an inducible promoter; these experiments are underway.

Construct stable cell lines expressing VP16-SXR under the control of an inducible promoter.

The pTet-On system from Clontech was utilized to construct representative models of ER+ and ER- stable lines with controlled, inducible expression of VP16-SXR. MCF7, T47D, MBMDA231 and MBMDA435 cells were transfected with the pTet-On plasmid, and cell lines stably expressing the reverse-tet repressor from the pTet-ON plasmid in the presence of doxycycline (dox) were identified. The candidate stable cell lines were further tested for background and inducibility by dox by transient transfection analysis. The dox-dependent pTet-on reporter, pTRE2-luc was transfected into cell lines and luciferase activity measured in the presence or absence of 1 μ g/ml Dox. Cell lines with inducibility ranging from 10 to 33-fold were identified and selected as the basis for the final cell line. These lines were selected both for high-level inducibility by dox and for low basal activity.

Before introducing the next construct, pTRE2-VP16-SXR into this background, we first examined the background inducibility of pTRE2-VP16-SXR in these cell lines to ensure that it was not significantly expressed in the absence of inducer. The T47D pTet-On stable lines showed the best inducibility of our breast cancer cell lines, hence we selected it for preliminary testing. We transfected pTRE2-VP16-SXR together with the SXR reporter tk-(CYP3A4)₃-luc (3) into candidate T47D pTet-On stable lines and measured luciferase activity in the presence or absence of dox. To our surprise, there was an approximately eight-fold stimulation of the reporter gene activity in the absence of dox that was dependent on pTRE2-VP16-SXR (Table 1). This level of SXR activity is enough to substantially blunt proliferation of cells compared with controls; hence, we conducted numerous experiments aimed at reducing this “leaky” control. The effector plasmid background was changed from pTRE2 to pBgal-basic, which contains a long poly A⁺ coding sequence upstream of the promoter, which is known to reduce or eliminate read-through transcription from upstream promoters. This was ineffective (Table 1). We also made several truncations in the 3' UTR of the VP16-SXR plasmid aiming to destabilize the mRNA and reduce background. This was also ineffective (Table 1). We also tried co-transfecting a plasmid encoding the tet repressor as recommended by Clontech without significant success.

Table 1 - Activation of tk(CYP3A4)₃-luc by pTRE2-VP16SXR in the presence or absence of dox. Columns 2,3,4 show strong background induction of the reporter in the absence of dox.

effector:	pTRE2	pTRE2-VP16SXR	shorter 3' VP16SXR	pBgal-VP16SXR
reporter:	CYP3A4-luc	CYP3A4-luc	CYP3A4-luc	CYP3A4-luc
<i>minus Dox</i>	0.64	4.3	5.2	4.2
<i>Fold induction</i>		6.7	8.1	6.6
<i>plus Dox</i>	0.64	76.8	61.3	78
<i>Fold induction</i>	1.0	120	95.8	121.8

We infer that the fundamental problem is that basal transcription from the minimal CMV promoters in the pTRE2 and pBgal basic vectors was sufficient to produce non-negligible amounts of the very potent and stable transcriptional activator VP16-SXR. This small amount of transcriptionally active SXR was able to bind to the CYP3A4-luc reporter, resulting in the background luciferase activity we detected, even in the absence of dox (Table 1).

In a further effort to make stable cell lines expressing VP16-SXR under the control of an inducible promoter, we are replacing the minimal CMV promoter in pTRE2 with the minimal

MMTV promoter that is known to have a very low background from basal transcription (4). In the event that this strategy does not work, we will switch from the pTet-On system to the ecdysone-inducible system (4).

Test whether the induction of VP16-SXR mimics the anti-proliferative activity of SXR activators.

We were unable to proceed with comparing proliferation of stable VP16-SXR cell lines with SXR activator-treated breast cancer cells due to problems with the VP16-SXR stable lines as described above. However, we were able to adapt a new method for examining the effects of SXR activators and constitutively active SXR (VP16-SXR) on the proliferation of breast cancer cells. This method utilizes the Vybrant CFDA SE Cell Tracer (Molecular Probes). Breast cancer cells are labeled with CFDA SE, which passively diffuses into cells and is colorless and non-fluorescent. When acetate groups on the dye are cleaved off inside cells, the dye becomes fluorescent. Excess label is then washed away and the retained fluorescence in labeled cells is inherited by daughter cells after cell division. These daughter cells have 50% of the fluorescence found in the parent cell. In this way, proliferation of the cell population can be measured by quantitating the total cell fluorescence over time compared with controls.

MCF7 cells were labeled with Vybrant CFDA SE and then transfected with VP16-SXR, VP16 or control vector. After several days, the cellular fluorescence of the transfected cells was measured. As shown in Figure 2, the VP16-SXR transfected cells retained more fluorescence than the VP16 or vector control cells meaning the VP16-SXR cells underwent fewer cell divisions than the control cells. Side by side with this experiment, MCF7 cells were also labeled with Vybrant CFDA SE and then treated with 10 μ M rifampicin. As expected from the results of previous proliferation assays, the potent SXR

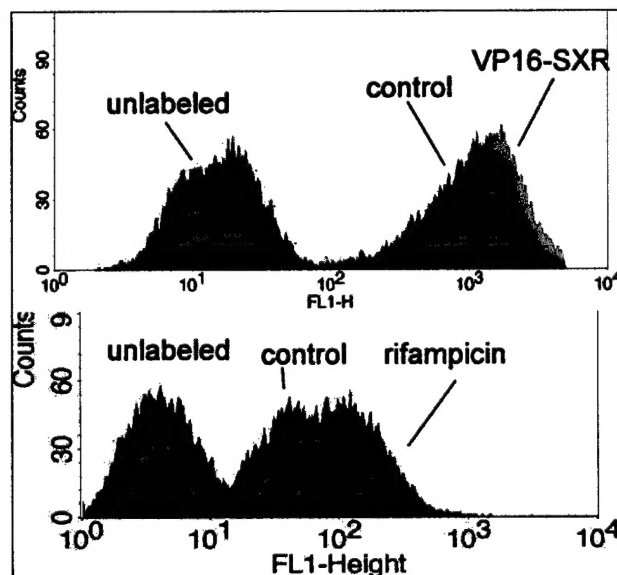


Figure 2 Lineage analysis of proliferation in cells transfected with VP16-SXR or control plasmids (top) or treated with rifampicin or solvent controls (bottom).

activator rifampicin was able to slow the growth of MCF7 cells compared to controls as measured by the increased fluorescence in the treated cells compared with control. The difference between transient transfection with VP16-SXR and rifampicin treatment probably derives from the number of affected cells. Virtually 100% of cells are responsive to rifampicin whereas only 10-15% are transfected and showing an effect by VP16-SXR. We conclude that the Vybrant CFDA SE method can be effectively utilized to measure the proliferation of breast cancer cells whether treated or transfected.

Test whether the inhibition of SXR activation with ET-743 blocks the anti-proliferative effects of SXR activators.

We applied to obtain the SXR antagonist ET-743 from its commercial manufacturer PharmaMar nearly six months ago. To date we have been unsuccessful. Although we hope that our application will be approved shortly, having another antagonist would be very useful. Interestingly, research in our other projects has identified certain polychlorinated biphenyls (PCBs) as novel SXR antagonists. Notably, we found that several non-planar PCBs could act as potent SXR antagonists (5). The most potent of these, PCB197, has a K_i of 0.6 μM , which establishes it as a reasonably potent antagonist. If it is not possible to obtain ET-743 in a timely fashion, we will utilize PCB197 to evaluate whether the anti-proliferative effects of SXR activators can be overcome with the antagonists.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of stable cell lines expressing pTet-ON, which induce the expression of target genes (other than VP16-SXR) in a robust manner without significant background.
- Constitutively active SXR (VP16-SXR) is able to slow the proliferation of breast cancer cells.
- A new method utilizing Vybrant CFDA SE lineage tracer can be used to measure the proliferation of breast cancer cells transfected with VP16-SXR or treated with SXR activators.
- SXR mRNA is expressed in ductal carcinomas but not in normal breast tissue.

REPORTABLE OUTCOMES

Presentations:

- May 7, 2002 – Seminar presentation at Long Beach VA Medical Center, Long Beach, CA
- January 23, 2003 – Seminar presentation at Symposium on Endocrine Disrupter Research, Matsuyama, Japan
- January 27, 2003 – Seminar presentation at Inoue Project Meeting on Endocrine Disrupter Research, Tokyo, Japan
- January 30, 2003 – Seminar presentation at the National Institute for Basic Biology, Okazaki, Japan
- Feb 13, 2003 – Seminar presentation at the Academy of Lifelong learning held at UCI, Irvine, CA
- Mar 31, 2003 – Seminar presentation in UCI course in Corporate and Translational Research, Irvine, CA

Cell lines: pTet-On stable MCF7, T47D, MBMDA231 and MBMDA435 breast cancer cell lines expressing the reverse tet repressor and highly inducible by doxycycline.

CONCLUSIONS

One of the major challenges in breast cancer research is to develop new chemotherapeutic and chemopreventative agents, particularly for non-estrogen dependent breast cancers. SXR activators were able to slow the proliferation of ER+ and ER- breast cancer cell lines in culture, and a constitutively active form of SXR was also effective at slowing breast cancer cell growth. Establishment of stable cell lines with controlled expression of VP16-SXR will reaffirm the results we have already seen in transiently transfected cells and will clearly establish the link between SXR activation and inhibition of tumor cell proliferation. Expression of SXR mRNA in ductal carcinomas but not in normal tissue could mean that the presence or absence of SXR is an important prognostic marker for the success of breast cancer treatment. Moreover, fully understanding the mechanisms through which SXR exerts its action in the next year of funding will provide opportunities for rational drug design and improvement of the efficacy of existing drugs that act through SXR.

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